Calcium Signaling and Cytotoxicity

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The divalent calcium cation Ca^{2+} is used as a major signaling molecule during cell signal transduction to regulate energy output, cellular metabolism, and phenotype. The basis to the signaling role of Ca^{2+} is an intricate network of cellular channels and transporters that allow a low resting concentration of Ca^{2+} in the cytosol of the cell ($[Ca^{2+}]_i$) but that are also coupled to major dynamic and rapidly exchanging stores. This enables extracellular signals from hormones and growth factors to be transduced as $[Ca^{2+}]_i$ spikes that are amplitude and frequency encoded. There is considerable evidence that a number of toxic environmental chemicals target these Ca^{2+} signaling processes, alter them, and induce cell death by apoptosis. Two major pathways for apoptosis will be considered. The first one involves Ca^{2+}-mediated expression of ligands that bind to and activate death receptors such as CD95 (Fas, APO-1). In the second pathway, Ca^{2+} has a direct toxic effect and its primary targets include the mitochondria and the endoplasmic reticulum (ER). Mitochondria may respond to an apoptotic Ca^{2+} signal by the selective release of cytochrome *c* or through enhanced production of reactive oxygen species and opening of an inner mitochondrial membrane pore. Toxic agents such as the environmental pollutant tributyltin or the natural plant product thapsigargin, which deplete the ER Ca^{2+} stores, will induce as a direct result of this effect the opening of plasma membrane Ca^{2+} channels and an ER stress response. In contrast, under some conditions, Ca^{2+} signals may be cytoprotective and antagonize the apoptotic machinery. — *Environ Health Perspect* 107(Suppl 1):25–35 (1999). *http://ehpnet1.niehs.nih.gov/docs/1999/Suppl-1/25-35kass/abstract.html*

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The divalent calcium cation Ca^{2+} has a unique position among cellular ions in higher organisms. In its insoluble form, it is the major structural constituent of bones and teeth, whereas in its soluble form Ca^{2+} plays important roles such as membrane stabilizer, cofactor for proteins, electric charge carrier, and diffusible intracellular messenger (*1,2*). These roles result partly from the unusual distribution of soluble Ca^{2+} in the intracellular and extracellular environments and partly from the unique ability of Ca^{2+} to interact with proteins. The total Ca^{2+} concentration in extracellular biologic fluids such as blood serum ranges from 1.6 to 2 mM (of which approximately 50% is bound to proteins and other constituents). Within cells, Ca^{2+} is bound primarily to phospholipids, proteins, and nucleic acids or sequestered in organelles; only 0.1% of the total cellular Ca^{2+} content is found free in the cytosol. Consequently, the cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$) is kept around 0.1 μM (Figure 1); therefore, only a minute fraction of the total cellular Ca^{2+} is available for performing its function as charge carrier or diffusible messenger.

The direct consequence of the unusual distribution of Ca^{2+} in higher organisms is that their cells are constantly exposed to an extremely large electrochemical gradient between extracellular and intracellular Ca^{2+} concentrations (mM versus sub-μM). However, cells are equipped with sophisticated transport and sequestration mechanisms enabling them to carefully maintain their Ca^{2+} levels. In addition, subtle changes in Ca^{2+} transport kinetics allow cells to rapidly alter $[Ca^{2+}]_i$ in both an amplitude- and frequency-encoded manner to induce controlled changes in metabolism and cellular phenotype. It is also not surprising that a perturbation of the mechanisms controlling cellular Ca^{2+} homeostasis and Ca^{2+} signaling processes, either through inherited genetic abnormalities or through exposure to drugs or environmental agents, is the basis for many diseases and other pathologic conditions. This review summarizes recent progress in understanding how alterations in Ca^{2+} signaling through drugs and toxic environmental agents can affect the survival and functioning of cells and, hence, lead to conditions such as cancer, diabetes, and other autoimmune diseases, and neurodegeneration.

Regulation of Ca^{2+} in Mammalian Cells

To keep from being flooded by Ca^{2+} from the extracellular milieu, cells have acquired during evolution sophisticated transport mechanisms that carefully control access of Ca^{2+} into the interior of a cell across the plasma membrane and redistribution of Ca^{2+} from the cytosol into intracellular organelles (Figure 1) (*2–5*).

Ca^{2+} Transport across the Plasma Membrane

Ca^{2+} gains access into cells across the plasma membrane primarily through a number of channels, some of which are tight control by receptors (receptor-operated Ca^{2+} channels), the potential across the plasma membrane (voltage-gated Ca^{2+} channels) and the content of intracellular Ca^{2+} stores (store-operated Ca^{2+} channels), whereas others appear to be nonselective leak channels (*3*). Ca^{2+} can also gain access into the interior of cells in exchange for Na^+ by way of the plasma membrane sodium–calcium exchanger (*6*). To counteract the continuous influx of Ca^{2+} into the cell, the plasma membrane contains a Ca^{2+}-ATPase-type pump (PMCA) that uses ATP-dependent phosphorylation of an aspartate residue to translocate Ca^{2+} from the cytosol to the extracellular environment (*7*). In most tissues this pump is activated by the Ca^{2+}-binding protein calmodulin, which enables it to respond readily to increases in $[Ca^{2+}]_i$ with an increased Ca^{2+} translocating activity.

Ca^{2+}-Binding Proteins

Once inside a cell, Ca^{2+} can either interact with so-called Ca^{2+}-binding proteins or

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Abbreviations used: BiP, immunoglobulin heavy chain binding protein; $[Ca^{2+}]_i$, cytosolic-free Ca^{2+} concentration; CICR, Ca^{2+}-induced Ca^{2+} release; ER, endoplasmic reticulum; GRP, glucose regulated protein; $InsP_3$, inositol 1,4,5-trisphosphate; $InsP_3R$, inositol 1,4,5-trisphosphate receptor; NF-AT, nuclear factor of activated T cells; PLC, phospholipase C; PMCA, plasma membrane Ca^{2+}-ATPase; SERCA, sarco/endoplasmic reticulum Ca^{2+}-ATPase; tBHQ, 2,5-di(*tert*-butyl)hydroquinone; TBT, tributyltin.

become sequestered into endoplasmic reticulum (ER), mitochondria, or nucleus. An increasingly large number of proteins have been ascribed to have Ca^{2+}-binding functions with varying binding affinities and capacities for Ca^{2+} (*2,4*). Some of the intracellular binding proteins such as calmodulin act as Ca^{2+} receptors. Through the Ca^{2+}–protein interaction and resulting conformational change within the target protein, Ca^{2+} signals can be effectively relayed and amplified. Other proteins appear to act as Ca^{2+} storing devices (e.g., the calsequestrin and calreticulin families).

Intracellular Ca^{2+} Sequestration by Organelles

The largest store of Ca^{2+} in cells is found in the endoplasmic and sarcoplasmic reticula (*4,8*), with local concentrations reaching millimolar levels (*9*). Such high concentrations are achieved within the ER through the action of the sarco–endoplasmic reticula Ca^{2+}-ATPase-type pumps referred to as SERCAs (*4*). Like their PMCA counterparts, SERCAs exist as different isoforms depending on tissue of origin and use ATP to translocate vectorially Ca^{2+} from the cytosol into the ER.

It is well known that mitochondria possess a high capacity to sequester Ca^{2+} (*10*); yet under physiologic conditions *in vivo,* total mitochondrial Ca^{2+} levels and free $[Ca^{2+}]$ reflect and parallel cytosolic $[Ca^{2+}]$ (*8,11,12*). However, under a pathologic situation in which cells are exposed to high levels of Ca^{2+}, mitochondria have been found to start sequestering significant amounts of Ca^{2+} (*13,14*). Mitochondria take up Ca^{2+} electrophoretically through a uniport transporter. Release of Ca^{2+} is accomplished by three different routes: - *a*) a reversal of the uniporter, *b*) an Na^+-dependent (or independent) exchanger, and *c*) through an inner mitochondrial membrane pore that is involved in a phenomenon known as inner mitochondrial membrane permeability transition.

The transport of Ca^{2+} across the nuclear membrane has been the subject of much controversy [for example (*15,16*)]. Ca^{2+} must gain access to the nucleus to alter the activity of several transcription factors as part of the phenotypic effects of Ca^{2+} signaling, and reports (*17,18*) have shown that a Ca^{2+} wave initiated in one part of the cytosol of a cell will readily move across the nucleus. In contrast, other laboratories, including our own, have shown that the movement of Ca^{2+} across the nuclear membrane may be restricted. Thus, despite the observation that even proteins readily permeate the nuclear membrane because of the presence of nuclear pores (*19*), the movement of Ca^{2+} across the nuclear envelope has been reported to require a SERCA-like pump (*20*). However, it remains unclear whether this active transport of Ca^{2+} is at the level of the pores or the envelope.

Ca^{2+} Signaling in Mammalian Cells

The presence in cells of intracellular Ca^{2+} stores, particularly within the ER, that are in rapid equilibrium with the cytosol is the basis of the Ca^{2+} signaling machinery. Mammalian cells respond to a complex array of phosphorylation events and diffusible messenger generation triggered by numerous hormones and growth factors with the production of controlled increases in $[Ca^{2+}]_i$. These increases result from the combination of Ca^{2+} release from intracellular stores in response to either the diffusible messenger inositol 1,4,5-trisphosphate ($InsP_3$) or a Ca^{2+} spike (Ca^{2+}-induced Ca^{2+} release [CICR]) and Ca^{2+} influx across the plasma membrane (Figure 2) (*21*). $[Ca^{2+}]_i$ is often observed to rapidly rise in one particular area within a

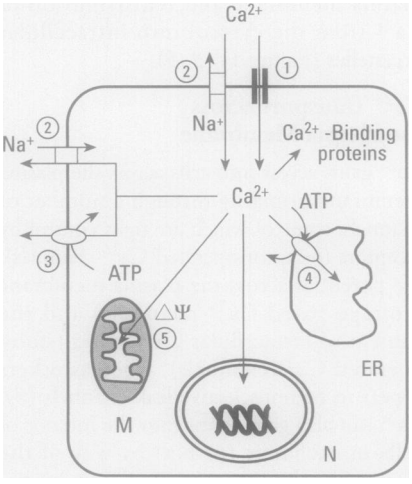

Figure 1. Schematic representation of the distribution of Ca^{2+} in cells. Abbreviations: ER, endoplasmic reticulum; M, mitochondria; N, nucleus, SERCA, sarcoplasmic–endoplasmic reticulum Ca^{2+}-ATPase. Two major routes for Ca^{2+} entry into cells exist, through Ca^{2+} channels (1) or in exchange for Na^+ via the sodium–calcium exchanger (2). Once inside the cell, Ca^{2+} can be translocated back to the extracellular environment, primarily by the action of the plasma membrane Ca^{2+}-ATPase (3) but also by the sodium–calcium exchanger (2). In addition, Ca^{2+} will interact with Ca^{2+}-binding proteins or become sequestered by the ER, M and N. The ER contains the SERCA, which translocates Ca^{2+} from the cytosol into the ER lumen (4), whereas mitochondria take up Ca^{2+} through their membrane potential (5). Further details are discussed in the text.

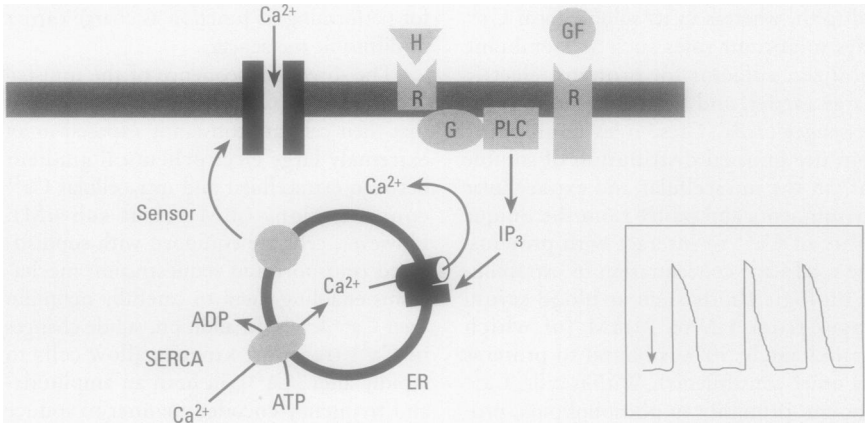

Figure 2. Ca^{2+} signaling in mammalian cells. Ca^{2+} signaling is initiated by the interaction of H, neurotransmitters or GF with their specific receptors. Abbreviations: ADP, adenosine diphosphate; ATP, adenosine triphosphate; ER, endoplasmic reticulum; G, G protein; GF, growth factor; H, hormones; $InsP_3$, inositol 1,4,5-triphosphate; PLC, phospholipase C; R, receptor; SERCA, sarcoplasmic–endoplasmic reticulum Ca^{2+}-ATPase. These receptors may be G protein coupled and activate PLC through the α subunit of the G protein or through direct coupling in the case of tyrosine kinase receptors for a number of growth factors. The action of PLC on phosphatidylinositol 4,5-bisphosphate leads to the formation of the diffusible messenger $InsP_3$ and the protein kinase C activator diacylglycerol (not shown). $InsP_3$ binds to its receptor located on the ER and opens a channel resulting in the discharge of ER Ca^{2+} into the cytosol. This in turn produces a decrease in ER Ca^{2+} content that is recognized by a yet poorly understood mechanism that triggers the opening of Ca^{2+} channels located in the plasma membrane and Ca^{2+} influx into the cell. The inset is a schematic representation of the changes in cytosolic Ca^{2+} concentration during Ca^{2+} signaling. Note that the increase in Ca^{2+} often occurs as a repetitive event with a defined amplitude and periodicity.

cell and then to rapidly spread as a Ca^{2+} wave across the entire cell. In the continuous presence of receptor agonist, the Ca^{2+} wave is often repeated at defined intervals, producing $[Ca^{2+}]_i$ oscillations. This is now viewed as a mechanism for providing the cell with a Ca^{2+} signal, the frequency and amplitude encoding of which depends on the intensity and nature of the stimulus. The cell appears to benefit in several ways from this type of Ca^{2+} signaling. First, the oscillating nature of the $[Ca^{2+}]_i$ allows a graded response. This is best exemplified by the enzyme Ca^{2+}/calmodulin kinase II, in which the kinase recruits increasing numbers of calmodulin molecules with increasing $[Ca^{2+}]_i$ oscillatory frequency until the enzyme becomes fully active (*22*). A second important facet of the oscillatory nature of Ca^{2+} signals is that it allows the signals to be relayed more efficiently into mitochondria (*12*). Finally, it is important to remember that a prolonged elevation of $[Ca^{2+}]_i$ will have detrimental effects on cell survival which leads to cell death by apoptosis or necrosis (*23,24*). Therefore, the frequency encoding of Ca^{2+} signals allows physiologic responses to occur without compromising cell survival.

The molecular basis of the Ca^{2+} oscillations has been the subject of numerous specialized reviews [for example (*5,21*)] and therefore will be only briefly summarized here. The initiator of the Ca^{2+} wave is $InsP_3$, which is formed by the action of phospholipase C (PLC) on the minor plasma membrane phospholipid, phosphatidylinositol 4,5-bisphosphate; the other product of this reaction is diacylglycerol, an activator of protein kinase C isoenzymes (Figure 2). Two major subfamilies of PLC exist, namely PLCβ, whose members are activated primarily by G protein coupled receptors, and PLCγ, whose members are controlled by protein tyrosine kinase receptors and protein tyrosine kinase-associated receptors. $InsP_3$, once formed, will readily diffuse away from the site of formation to bind to Ca^{2+}-release receptor channels, the inositol 1,4,5-trisphosphate receptors ($InsP_3Rs$) (*4,25*). Three forms of $InsP_3Rs$ (types 1, 2, and 3) have been characterized by cDNA cloning. Most cells possess at least one form of $InsP_3R$ and many express all three subtypes. $InsP_3Rs$ are localized primarily on the ER (or specialized subareas of ER) as tetramers of large subunits and resemble in their structure and molecular organization the ryanodine receptors, the voltage- or Ca^{2+}-sensing Ca^{2+}-release channels responsible for CICR (*4*).

The quantal discharge of the sequestered Ca^{2+} from ER by the action of IP_3 that leads to the formation of a Ca^{2+} wave can be explained by a number of complex models [for example (*21*)]. Once released into the cytosol, Ca^{2+} may be recycled by re-uptake into ER by the SERCAs, transient sequestration by mitochondria and extrusion from the cell by the PMCAs. In fact, the latter appears predominant in cells such as hepatocytes (*26,27*), and this explains why Ca^{2+} oscillations are only short lived in the absence of extracellular Ca^{2+} [for example (*28*)]. The refilling of ER stores therefore requires replenishing; this is achieved through a phenomenon known as store-operated Ca^{2+} influx (*29*). Store-operated Ca^{2+} influx, which was first coined capacitative Ca^{2+} entry, was discovered through a series of experiments conducted by Putney and co-workers (*30,31*).

The essence of the system is that a sensing mechanism is present in the ER (or the portion associated with the $InsP_3$-sensitive Ca^{2+} store) that detects the decrease in ER Ca^{2+} content caused by the discharge into the cytosol through $InsP_3R$ channel openings. This sensing mechanism then sends a signal to plasma membrane channels that are distinct from classic receptor-operated and voltage-gated channels and that are highly specific for Ca^{2+} (Figure 2) (*29,32,33*). Whether the signal for Ca^{2+} channel opening is a diffusible messenger (Ca^{2+} influx factor, cGMP) or occurs through direct coupling remains controversial. An important point to note in this context is that this signal may be long lived, and therefore chemicals that inhibit SERCAs such as thapsigargin and 2,5-di(*tert*-butyl)hydroquinone (*34,35*) will cause a prolonged stimulation of Ca^{2+} entry into cells as a result of ER Ca^{2+} release. This leads to a sustained elevation of $[Ca^{2+}]_i$ (Figure 3) and thereby to an array of pathologic conditions.

Ca^{2+} and Cytotoxicity

Given the complexity of the regulation of cellular Ca^{2+} and Ca^{2+}-signaling processes, it is not surprising that disruption of these control mechanisms has been linked to the pathogenesis of diseases and cytotoxic events. Indeed, work from several laboratories including our own showed in the 1980s that a perturbation of Ca^{2+} homeostasis is a common and final event responsible for drug-induced cell death (*23,36–40*). The overall picture that emerged from this work was that the perturbation of Ca^{2+} homeostasis was caused by the inhibition of Ca^{2+} transport mechanisms including the PMCAs and SERCAs by the cytotoxic chemicals or their metabolites. Consequently, the injured cells are exposed to a prolonged elevation of $[Ca^{2+}]_i$ that in turn activates several catabolic processes catalyzed by Ca^{2+}-activated proteases (calpains), phospholipases, and endonucleases.

A confusion arose in our understanding of chemical-induced cell death when in the late 1980s it became clear that this type of cell death could occur not only by necrosis but also by another form of cell death known as apoptosis. Apoptosis is a form of programmed cell death occuring during organogenesis and organ remodeling and

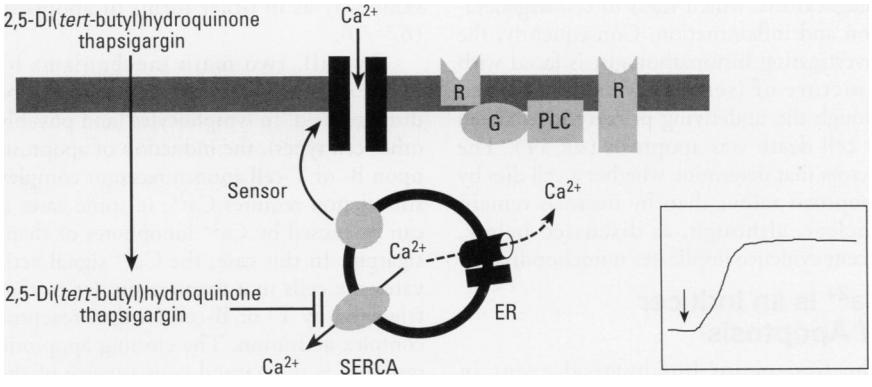

Figure 3. Changes in cellular Ca^{2+} induced by SERCA inhibitors. Abbreviations: ER, endoplasmic reticulum; G, G protein; PLC, phospholipase C; R, receptor; SERCA, sarcoplasmic–endoplasmic reticulum Ca^{2+}-ATPase. The inhibition of SERCA by agents such as 2,5-di(*tert*-butyl)hydroquinone or thapsigargin results in the depletion of the ER Ca^{2+} store. As discussed in the text, this is recognized by the cell and results in the opening of specific plasma membrane Ca^{2+} channels. Unlike signaling through $InsP_3$ (Figure 2), SERCA inhibition often leads to a prolonged Ca^{2+} influx response producing a sustained increase in the cytosolic Ca^{2+} concentration (inset) and, in many cell types, death by apoptosis.

in adult life to modulate the immune system or to kill transformed and virally infected cells (*41–43*). Cells undergoing apoptosis show well-defined morphologic and biochemical changes including cellular and nuclear shrinkage, condensation, margination and fragmentation of chromatin, changes in plasma membrane architecture, and intracellular proteolysis (*42,44,45*). Enormous progress in our understanding of the molecular events involved in apoptosis has been achieved recently, and it is not surprising that Ca^{2+} has been found to play a pivotal role in this form of cell death.

Although necrosis and apoptosis initially were described as two fundamentally different forms by which a cell can die, an intense and often confusing debate currently prevails about whether this position is still tenable or whether we are dealing with a continuum of overlapping mechanisms of cell death (*46–48*). For instance, toxic chemicals that generate oxidative stress or induce a pathologic increase in cellular calcium levels can kill their target cells by either necrosis or apoptosis, depending on the degree of exposure (*40,46,48,49*). Several important features such as plasma membrane blebbing and mitochondrial damage are common to both apoptosis and necrosis. Also, several reports have shown that the product of the antiapoptotic gene *bcl-2* protects cells not only from apoptosis but also from necrosis (*50–53*). Therefore, the boundaries between apoptosis and necrosis may not be as distinct as often assumed. To add further to the confusion, it is also clear that when apoptosis occurs at an excessive level, the dying cells can no longer be removed in an orderly fashion by phagocytosis, which leads to cell degeneration and inflammation. Consequently, the investigating histopathologist is faced with a picture of (secondary) necrosis even though the underlying primary mechanism of cell death was apoptosis (*47,54*). The factors that determine whether a cell dies by apoptosis rather than by necrosis remain unclear, although, as discussed below, recent evidence implicates mitochondria.

Ca^{2+} Is an Inducer of Apoptosis

The first major biochemical event in apoptosis to be reported was that glucocorticoid-triggered apoptosis of thymocytes involved degradation of the nuclear DNA to nucleosomal- and oligonucleosomal-sized fragments by a specific endonuclease (*55*). When resolved by agarose gel electrophoresis, the nuclear DNA had a ladderlike appearance. Shortly afterward, Cohen and Duke (*56*) showed that calcium ions induced in isolated thymocyte nuclei the same pattern of DNA fragmentation through the activation of a Ca^{2+}- and Mg^{2+}-dependent endonuclease. This provided us with the first mechanistic clue to the biochemical events taking place during apoptosis. The subsequent dissection of the mechanism of glucocorticoid-induced apoptosis revealed that extracellular Ca^{2+} was necessary for cell death to occur (*57*). More direct evidence for a causal relationship between Ca^{2+} and apoptosis was presented when it was found that the Ca^{2+} ionophore A23187 could induce apoptosis in thymocytes (*58,59*). These observations led to the proposal of a model in which the Ca^{2+}- and Mg^{2+}-dependent endonuclease is a universal effector of apoptotic cell death through an increase in $[Ca^{2+}]_i$.

The model, although very attractive, was soon challenged when it became clear that DNA degradation to oligonucleosomes is often a relatively late event (*60*) in apoptosis or may even be completely absent in some cases (*61*). In addition, the cloning of the *C. elegans* gene *ced-3* led to identification of a novel family of cysteine proteases (caspases) that cleave their protein substrates at specific aspartate residues. Caspases are now accepted to be the general intracellular orchestrators of apoptosis and are responsible for most if not all biochemical and morphologic features of apoptosis (*62–66*). This led to reinvestigation of how Ca^{2+} signals can induce apoptosis and, not surprisingly, recent evidence shows that caspases are the effectors of Ca^{2+}-induced apoptosis in the same way as in other forms of apoptosis (*67–70*).

Overall, two main mechanisms by which Ca^{2+} can induce apoptosis must be distinguished. In lymphocytes (and possibly other cell types), the induction of apoptosis upon B- or T-cell antigen receptor complex stimulation requires Ca^{2+}; in some cases it can be caused by Ca^{2+} ionophores or thapsigargin. In this case, the Ca^{2+} signal activates the cells in a manner similar to that triggered by T- or B-cell antigen receptor complex activation. The ensuing apoptotic response is the natural consequence of the cell activation process rather than being due to a direct apoptotic effect of Ca^{2+} alone. Consequently, the apoptotic effect of Ca^{2+} should be regarded as *indirect*. In most cases, however, Ca^{2+} will trigger apoptosis in a manner that is *directly* related to a change in cellular Ca^{2+} levels.

Ca^{2+}-Induced Apoptosis: Direct Mechanisms

To investigate the role of Ca^{2+} signaling in apoptosis, we used the SERCA inhibitors tBHQ and thapsigargin (*71*). By inhibiting the pump responsible for translocating Ca^{2+} from the cytosol into the ER, these compounds induce rapid release of the ER Ca^{2+} stores in the absence of $InsP_3$ formation (*34,35*) and as a direct consequence of ER Ca^{2+} pool depletion, also the store-dependent Ca^{2+} influx response (Figure 3) (*29,33,72*). We found that thymocytes exposed to thapsigargin or tBHQ rapidly became apoptotic (*71*). The apoptotic response was abrogated in the absence of extracellular Ca^{2+}. Incubating thymocytes in nominally Ca^{2+}-free medium (i.e., in the absence of added Ca^{2+} but without the need for Ca^{2+} chelators such as EGTA) completely abrogated thapsigargin-induced apoptosis. This demonstrates that in thymocytes the apoptotic effect of SERCA inhibitors depends strictly on Ca^{2+} influx rather than the result of total cellular Ca^{2+} chelation or ER Ca^{2+} store depletion. Using excess Ca^{2+} chelators is an approach often adopted It is, however, limited by nonspecific effects resulting from total cell Ca^{2+} chelation (*73*).

SERCA inhibition is a mechanism used by a number of toxic chemicals. We found that the immunotoxic environmental contaminant tributyltin (TBT) induces thymocyte apoptosis through this very mechanism (*74*). TBT is a member of the highly toxic organotin family and has been extensively used in marine paints to prevent barnacles from attaching to marine vessels. Its use has raised much concern because of its highly cytotoxic properties, especially toward fish and mammals, which induce thymocyte and lymphocyte apoptosis (*75,76*). TBT induces apoptosis by inhibiting SERCA, which triggers release of ER Ca^{2+} and activation of the store-dependent Ca^{2+} influx. As a result of the prolonged inhibition of SERCA, the activation of the influx pathway leads to a massive accumulation of Ca^{2+}, which is worsened by the additional inhibition of the PMCA, and subsequent death of thymocytes by apoptosis (Figure 4) (*74*). A similar mechanism has been reported for organotin-induced apoptosis of PC12 cells (*77*). Consequently, inappropriate use of the cell's Ca^{2+} signaling machinery is an important mechanism by which environmental toxins like TBT induce apoptosis.

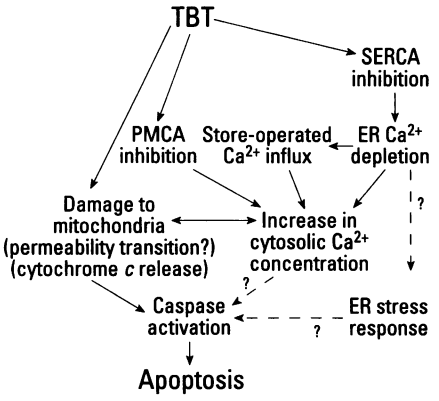

Figure 4. Schematic representation of the events that lead to apoptosis in thymocytes exposed to the environmental contaminant tributyltin. ER, endoplasmic reticulum; PMCA, plasma membrane Ca^{2+}-ATPase; SERCA, sarcoplasmic–endoplasmic reticulum Ca^{2+}-ATPase; TBT, tributylin. Details are discussed in the text.

Similarily, Ca^{2+} signaling processes may be targeted by toxic metals to cause a range of perturbations (*78–82*). For instance, inorganic mercury opens L-type Ca^{2+} channels and produces a sustained elevation of $[Ca^{2+}]_i$ and death of PC12 cells (*82*).

Our knowledge about the mechanism by which an increase in $[Ca^{2+}]_i$ leads to apoptosis is still fragmentary. A likely target for Ca^{2+} is the mitochondrion. As previously mentioned, mitochondria readily accumulate Ca^{2+} from cytosol to transduce the Ca^{2+} signal to a number of Ca^{2+}-activated dehydrogenases that participate in energy production. Mitochondria have also been postulated to play a role in buffering cytosolic Ca^{2+} (*10*). Indeed, exposing hepatocytes to SERCA inhibitors results in the rapid accumulation of Ca^{2+} by mitochondria (*14*), and recent evidence indicates that the spatial proximity between mitochondria and ER Ca^{2+} stores plays a critical role in this response (*83*).

Mitochondria and Apoptosis

Mitochondria are particularly vulnerable and a number of apoptotic stimuli including prooxidants and Ca^{2+} can induce a stress response known as inner membrane permeability transition. Mitochondrial permeability transition involves the opening of a pore that is made of a large proteinaceous complex comprising, among others, the voltage-dependent anion channel (VDAC), also known as porin, adenine nucleotide translocator, mitochondrial cyclophilin D, the peripheral benzodiazepine receptor, hexokinase, creatine kinase, and possibly also Bax (*84–86*). The pore complex has been localized to the contact sites between the inner and outer mitochondrial membranes. However, the molecular details of the association of the different components of the pore are still unclear although the conditions that trigger pore opening have been well defined. The pore behaves as a voltage-operated channel that becomes activated by high-matrix Ca^{2+}, oxidative stress, pyridine nucleotide oxidation, thiol oxidation, alkalinization, and low transmembrane potential. Initially, rapid and stochastic opening and closing of the pore is observed (*87,88*). This, however, rapidly develops into persistent pore opening, allowing not only Ca^{2+} but also low molecular weight matrix components ($M_r < 1500$) to escape rapidly from mitochondria (*89*). At this stage the opening of the pore is still reversible by agents such as cyclosporin A (*89*). How the transition occurs from an initially VDAC to a megachannel is presently unclear.

Mitochondria and pore opening play pivotal roles in cytotoxicity. We have previously reported that pore opening is an important event in prooxidant injury in hepatocytes (*13*). Pore opening occurred as a result of enhanced Ca^{2+} sequestration by and oxidative damage to the mitochondria. Inhibiting pore opening with cyclosporin A prevents or delays the onset of cell death, depending on the type of prooxidant used. In this experimental system of prooxidant injury, cell death occurred very rapidly with substantial cell swelling, suggesting that the cells died by necrosis rather than apoptosis.

There is no doubt that mitochondria play an important role in apoptosis. Yet, morphologically, they remain essentially intact during the initial stages of apoptosis (*42,43,60*). Similarily, mitochondria have been reported to function normally during the early stages of the process (*90*) even though a decrease in mitochondrial transmembrane potential has been observed in some cases (*91*). These observations suggesting that mitochondria are spared during apoptosis are difficult to reconcile with a recently proposed model that the permeability transition is a general mechanism of apoptosis (*91*). Because of its nature, mitochondrial permeability transition occurs with severe swelling and complete loss of cellular energy (*13,92,93*). In fact, recent work by Leist and co-workers (*94*) and Tsujimoto (*95*) has demonstrated that the apoptotic program necessitates cellular ATP, and these authors have suggested that ATP acts as a major decision switch between apoptosis and necrosis. Furthermore, our own results have shown that ATP is necessary for nuclear chromatin condensation to occur during apoptosis (*96*).

How apoptotic Ca^{2+} signals interact with mitochondria is unclear. Release of ER Ca^{2+} and stimulation of store-dependent Ca^{2+} entry lead to the rapid accumulation of the cation by mitochondria (*14*). If excessive, the result is permeability transition, as shown in isolated mitochondria (*89*) and intact cells (*97*). Permeability transition could occur through direct interaction with the metal-binding site of the pore (*89*), inhibition of respiration by NO (nitric oxide) through Ca^{2+} activation of the mitochondrial NO synthase (*98*), oxidative stress imposed by the loss of glutathione during apoptosis (*99,100*), superoxide anion production triggered as a result of cytochrome *c* loss (*101*), or a combination of several of the above events. However, Waring and Beaver (*97*) have also shown that very low concentrations of thapsigargin can induce apoptosis in the absence of detectable changes in mitochondrial morphology or transmembrane potential.

An alternative explanation for the contribution of mitochondria to apoptosis is that apoptotic stimuli induce mitochondria to release cytochrome *c* into the cytosol to activate caspases. This model currently is receiving rapidly increasing experimental support. The first indication of this pathway emerged when it was found that Jurkat T-lymphocytes showed a decrease in mitochondrial respiration during CD95-induced apoptosis. The defect in respiration was attributed to a loss of cytochrome *c* from mitochondria as respiration was reinstated by the supply of exogenous cytochrome *c* (*102*). Other investigators observed that cytochrome *c* is released from mitochondria in response to certain apoptotic stimuli (*103–105*). The released cytochrome *c* induces formation of a caspase-activating complex in the presence of dATP (*106,107*) that comprises procaspase-9 bound to Apaf-1 (the mammalian homolog of *Caenorhabditis elegans* CED-4) through a homophilic interaction involving caspase recruitment domain motifs. The proteolytic activation of the proform of caspase-9 to its active form occurs through an intrinsic mechanism and is followed by the downstream proteolytic activation of pro-caspase-3 and pro-caspase-7 (*108*). Recently we have found that microinjection of cytochrome *c* into cells is sufficient to induce apoptosis (*109*).

Several mechanisms have been proposed to account for the loss of cytochrome *c* from mitochondria in apoptosis. *In vitro* studies have shown that cytochrome *c* release can be induced by agents that cause permeability transition, possibly as a result of disruption of the outer membrane by the swelling associated with permeability transition (*110,111*). However, other reports clearly demonstrate that cytochrome *c* is mobilized from mitochondria prior to or in the complete absence of permeability transition (*105,112*) in a mechanism that may involve Bax, a member of the proapoptotic Bcl-2 family (*113,114*). Bcl-2 prevents cytochrome *c* release (*105*), possibly through its interaction with Bax. More recently a caspase-8-mediated cleavage product of the BH3-domain-containing Bid has been reported to efficiently release cytochrome *c* from mitochondria, also in the absence of permeability transition (*115,116*).

Whether the induction of apoptosis by Ca^{2+} signals requires release of cytochrome *c* is not yet known. The data by Waring and Beaver (*97*) suggest that at least permeability transition may not be necessary for Ca^{2+}-induced apoptosis. The reported antiapoptotic effects of Bcl-2 on Ca^{2+}-induced apoptosis are difficult to interpret because Bcl-2 prevents both cytochrome *c* release (*105*) and permeability transitions in response to Ca^{2+} accumulation (*91, 117*), as well as other effects on Ca^{2+} signaling (*118–120*). Interestingly, Bax recently has been detected in enriched fractions of the pore complex (*121*). Thus, the dual effect that Bcl-2 has on mitochondria—preventing cytochrome *c* release as well as permeability transition—suggests the existence of some form of functional or spatial relationship between cytochrome *c* release and permeability transition.

Ca^{2+}-Activated Proteases and Apoptosis

Many recent studies of apoptosis have focused on caspases. However, there is considerable evidence that additional proteases, including serine proteases [for example (*60,122–124*)] as well as members of the calpain family of Ca^{2+}-activated proteases (*125,126*), participate in apoptosis. Results of inhibitory studies indicate that the contribution of calpains to apoptosis appears to be limited to certain cell types such as thymocytes (*126*), monocytic U937 cells (*125*), cardiac myocytes (*127*), and neuronal cells (*128–130*).

The cellular targets for calpains in apoptosis are not well known. We know that fodrin is cleaved by calpains during tumor necrosis factor-induced apoptosis in U937 cells (*125*) and a Ca^{2+}-activated proteolytic system that cleaves lamins exists in thymocyte nuclei (*131*).

Recently, calcium has been found to increase caspase-3 activity in a cell-free system when added to nonapoptotic cell cytosol and to elicit nuclear morphologic changes and DNA fragmentation (*132*). However, the Ca^{2+}-induced processing and activation of pro-caspase-3 to caspase-3 did not involve calpains but an *N*-tosyl-L-phenyl chloromethyl ketone-sensitive serine protease. These findings may help not only to uncover the up-to-now elusive mechanism of caspase activation in Ca^{2+}-induced apoptosis but also to position more precisely the role of serine proteases in apoptosis.

Ca^{2+} Pool Emptying versus $[Ca^{2+}]_i$ Increase

Several studies have reported that the removal of extracellular Ca^{2+} did not prevent apoptosis induced by thapsigargin. Instead, a much closer correlation between ER Ca^{2+} pool emptying and apoptosis was observed (*133–137*). Evidence for a causal relationship between Ca^{2+} store emptying and apoptosis is based on several observations, particularly the ability of Bcl-2 to antagonize ER Ca^{2+} pool emptying (*120, 133,138*), the antiapoptotic effect of the ER Ca^{2+}-storing protein calreticulin (*139,140*), and the ability of high extracellular Ca^{2+} to restore Ca^{2+} pools thereby preventing apoptosis (*137*).

Depletion of ER Ca^{2+} stores triggers a stress condition reflected in a shutdown of both protein (*141,142*) and phosphatidylserine syntheses (*143,144*) and the transcriptional upregulation of several ER stress proteins that are also chaperones, including HSP70 (*145*), BiP (immuno globulin heavy chain binding protein)/GRP78 (glucose-regulated protein 78), GRP94, and ERp29 (*145–149*). The induction of the ER stress proteins following ER Ca^{2+} store emptying requires among others the transcription factors CBF/NF-Y, EGR-1, and YY1, which are activated under these conditions and interact with regulatory elements such as CCAAT (*147,150–152*). The significance of the stress response in the context of apoptosis is presently unclear, although recent evidence suggests that some of the ER stress proteins have a cytoprotective function rather than being proapoptotic (*153–156*). Interestingly, BiP/GRP78 is also a major Ca^{2+} storage protein, with approximately 25% of the ER Ca^{2+} store being bound to this protein (*157*). Together, the evidence indicates that ER Ca^{2+} pool emptying by SERCA inhibitors and Ca^{2+} chelators induces ER stress and apoptosis in numerous cell systems. However, we still know very little about how this stress response leads to apoptosis.

Ca^{2+}-Induced Apoptosis: Indirect Mechanisms

A major feature of the immune system is its plasticity, which enables it to respond rapidly to invading organisms and foreign, infected, or transformed cells. Clonal expansion of T lymphocytes that recognize specific antigens occurs through stimulation of the T-cell antigen receptor complex with signaling through $p21^{ras}$ and Rac and through phospholipase γ1. The latter enzyme mediates the release of $InsP_3$, Ca^{2+} mobilization, and downstream events involving calcineurin and nuclear factor of activated T cells (NF-AT). Once the infection has been overcome and the foreign or transformed cells eliminated, clonal expansion must be reversed and the T-lymphocyte elimination process occurs by apoptosis. Recent work has shown that the apoptotic response is effected by Ca^{2+} and calcineurin-mediated upregulation of CD95 (Fas, APO-1) ligand and TRAIL (APO-2 ligand), which in turn activate their death receptors and induce apoptosis (*158–160*). Consequently, Ca^{2+}-mediated apoptosis of lymphocytes does not appear to involve a direct apoptotic effect of Ca^{2+} but instead is indirectly mediated through upregulation of cytokines that activate plasma membrane death receptors.

Is Ca^{2+} Involved in All Forms of Apoptosis?

Many stimuli induce apoptosis in the absence of any detectable changes in Ca^{2+} fluxes or $[Ca^{2+}]_i$, and therefore no direct role of Ca^{2+} signaling is apparent. However, over the past few years, a number of reports have appeared in which components of the Ca^{2+} homeostatic and signaling machinery have been identified as important regulators of apoptosis. For example, ectopic expression of the Ca^{2+}-binding protein calbindin-D28K provides protection against a number of apoptotic stimuli (*161,162*). Similarily, the expression of $InsP_3R3$ in thymocytes, B and T cells is increased manyfold during apoptosis (*163*). Prevention of this expression by transfecting S49 cells with an $InsP_3R3$ antisense

plasmid antagonized glucocorticoid-induced apoptosis. More recently, T cells deficient in $InsP_3R1$ were found to be resistant to a wide variety of apoptotic stimuli including glucocorticoids, T-cell antigen receptor complex stimulation, ionizing radiation, and CD95 (*164*); CD95-induced myocyte toxicity has been shown to require $InsP_3R$ (*165*). Also, Ca^{2+}/calmodulin-dependent kinase II is stimulated by tumor necrosis factor and ultraviolet light, and inhibition of the kinase activity was found to prevent apoptosis (*166*). Similarily, the recently discovered Ca^{2+}/calmodulin-dependent kinase DAP-kinase induces apoptosis when overexpressed and, conversely, cells transfected with a catalytically inactive form of this enzyme are resistant to interferon-γ induced apoptosis (*167*). These observations indicate a more general participation of the Ca^{2+} signaling machinery in apoptosis than was previously thought. However, the molecular aspects of this participation are yet to be identified.

Ca^{2+} as an Antiapoptotic Signal

Most research on Ca^{2+} has focused on its apoptotic properties. Yet, in several models of apoptosis, Ca^{2+} has the opposite effect and can prevent apoptosis. Most striking is the case of neurons that undergo apoptosis upon withdrawal of nerve growth factor. Depolarization of the cells with K^+ to open voltage-operated Ca^{2+} channels provides the neurons with a survival signal (*168*). These observations led to the postulation of the Ca^{2+} set point hypothesis in which a minimum $[Ca^{2+}]_i$ is required to maintain neuron viability (*11,169*). When $[Ca^{2+}]_i$ moves below or above this set point, apoptosis is rapidly induced. The protective effect of depolarization-induced Ca^{2+} channel opening on neuronal cells is mimicked by thapsigargin (*170*).

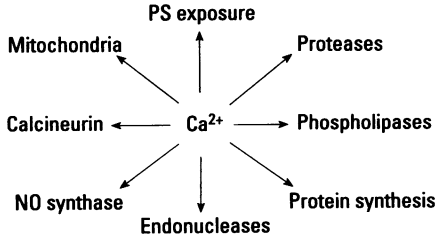

Figure 5. Some of the intracellular targets in Ca^{2+}-induced cytotoxicity. Abbreviation: PS, phosphatidylserine.

Ca^{2+} also protects against apoptotic signals in other cell types including hematopoietic cells, macrophages, myeloid leukemia cells, and neutrophils (*171–176*).

The mechanism responsible for the protective action of Ca^{2+} is unclear. In neurons, protection requires Ca^{2+} influx and appears to be mediated by calmodulin (*177*). The antiapoptotic effect of Ca^{2+} coincides with the prevention of caspase activation (*178*). Also, Lotem and Sachs (*175*) found that extracellular Ca^{2+} was required in M1 myeloid leukemia cells and that Ca^{2+} prevented the activation of caspases. The protective effect of Ca^{2+} was in this case abolished by cyclosporin A, suggesting a role for calcineurin in the mechanism of Ca^{2+} action. These findings suggest, therefore, that transcriptional activation and *de novo* synthesis of antiapoptotic signals are important. An additional possible explanation is that Ca^{2+} influx keeps the ER Ca^{2+} pool filled, which prevents the complete ER Ca^{2+} pool depletion by the apoptotic stimulus and the ER stress response.

Conclusions and Perspectives

There is little doubt that Ca^{2+} signals participate in apoptosis. Given the complexity of Ca^{2+} signaling, it is hardly surprising that identifying the exact mechanism of Ca^{2+}-induced apoptosis has proved to be a major challenge. Not only are we faced with a large number of potential cellular targets for Ca^{2+} action (Figure 5) but also numerous stimuli inducing Ca^{2+} signals activate other cell signaling pathways. The many possibilities of cross-talk between the different signals add to the complexity of the situation. This problem is particularly difficult when dealing with cytotoxic environmental chemicals such as prooxidants that can induce apoptosis by affecting signaling pathways at multiple levels (*179*). To add further to the complexity, Ca^{2+} can, under some circumstances, show antiapoptotic properties. Interestingly, this dual feature is shared by several inducers of apoptosis, notably tumor necrosis factor. A possible consequence of the complexity of the interactions with the Ca^{2+} signaling machinery is that there may be no reliable universal parameter that could be used to detect and assess the health risk associated with exposure to cytotoxic environmental chemicals.

Two likely targets for the apoptotic effects of Ca^{2+} signals have been identified over the past few years (Figure 6). One is

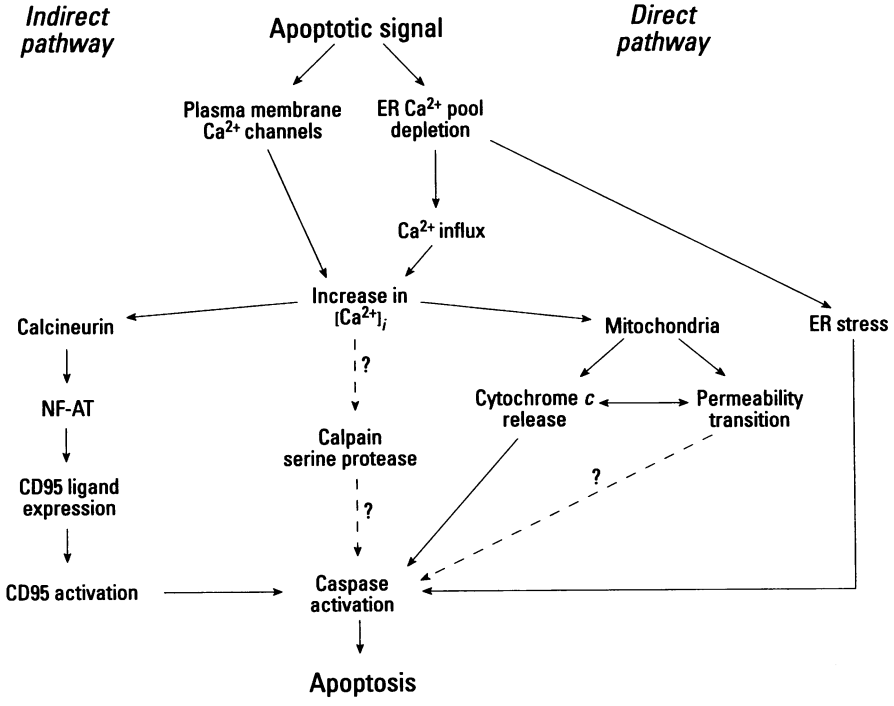

Figure 6. Proposed mechanisms of apoptosis induced by Ca^{2+} signals. Abbreviations: ER, endoplasmic reticulum; NF-AT, nuclear factor of activated T cells. Two pathways are recognized, one that is indirect and requires the activation of death receptors such as CD95 (Fas, APO-1) and a second pathway where Ca^{2+} is the direct effector of apoptosis. In the latter case, the two major targets are the mitochondria and the ER. Further details are given in the text.

mitochondria. There is rapidly increasing evidence that these play a major role in some forms of apoptosis. Apoptosis may be triggered through cytochrome *c* release and possibly through (limited?) permeability transition. The questions of which mechanism is used by Ca^{2+} and how this is linked to caspase activation must be addressed. The other target is the ER, where the emptying of its Ca^{2+} content induces a form of cell stress that ultimately leads to apoptosis. Again, further work is required to interpret the signals that interconnect ER stress, caspase activation, and apoptosis. The etiology of many diseases such as cancer, neurodegeneration, diabetes, and autoimmune diseases has been linked to an improper regulation of apoptosis, with considerable evidence for the involvement of cytotoxic environmental chemicals in some of these diseases. There is much hope that a better understanding of the molecular events in apoptosis will improve chances to develop better preventive and therapeutic strategies to control apoptosis and hence the development of disease.

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